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MacNeil, C

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Calum MacNeil[1], Mark Briffa[2]

Authors' addresses:

[1]Brookvale Cottage, Main Road, Union Mills, Isle of Man IM4 4AJ. British Isles.

[2]School of Biological and Marine Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA. U.K.

Correspondence: calummac73@hotmail.com

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Abstract

Non-consumptive effects (NCEs) of predators – so called 'fear' responses – encompass costly antipredator behaviours, such as reduced feeding efficiency. NCEs can influence prey population dynamics and community structure, if prey are 'keystone' species such as *Gammarus* spp. amphipod 'shrimps'. These freshwater macroinvertebrates have the ecosystem functional role of shredding fallen leaf litter, making it accessible to other taxa. Across Europe, the invasive predatory 'killer shrimp' *Dikerogammarus villosus* is replacing resident *Gammarus* spp., potentially threatening this vital ecosystem function. While predation (consumptive effects (CEs)) of this invader has been well studied, for the first time we test whether NCEs can be evident in prey only exposed to *D. villosus* presence and whether this could potentially impact on the prey's functional role. In mesocosms, exposure to constrained *D. villosus* did not result in mortalities of any of three *Gammarus* prey species but the leaf shredding efficiencies of all prey were significantly reduced compared to a control treatment. This clear NCE has the potential to propagate through the ecological community via decreased energy processing. This study demonstrates the potential for fear of invasive predator presence alone to impact on ecosystem function.

Key words: Consumptive effect (CE), *Dikerogammarus*, killer shrimp, non-consumptive effect (NCE), shredders

Introduction

The 'naive prey' hypothesis posits that resident prey lacking shared evolutionary history with invaders can be heavily predated because of ineffective anti-predator responses [1]. While effective anti-predator responses reduce predation rates (i.e. consumptive effects, CEs), tactics such as predator avoidance by prey can be costly resulting in non-consumptive, or 'trait mediated' effects (NCEs) such as reduced foraging efficiency [2]. Described as 'fear' responses, these tactics used by individuals can influence population densities and dynamics through impacts on survival and reproduction [2]. If prey species perform key ecosystem functions such as energy processing, NCEs may propagate further through ecological communities. Thus, there may be cumulative effects of NCEs alongside CEs that impact the structure of resident communities subject to invasion by predatory species [3].

Studies on the impacts of biological invasion have traditionally focussed on competition, predation and biodiversity loss [4]. Within three decades, the Ponto-Caspian amphipod *Dikerogammarus villosus* has invaded the freshwaters of nearly twenty European countries [5]. *D. villosus* is a voracious predator consuming a vast range of macroinvertebrate taxa across different trophic groups, it can be super-abundant within invaded sites, dominating assemblages and its invasion is linked to local extinctions [5]. In the Netherlands, declines of two resident amphipods, the native *Gammarus duebeni* and a previous invader *G. tigrinus*, shortly after the arrival of *D. villosus*, have been attributed to predation by the invader [6] and another amphipod, *G. pulex*, may also be under threat of species replacement by *D. villosus* in areas of Central Europe and the U.K. [5]. Nevertheless, *D. villosus* and its resident prey can also co-exist, both during and following range expansion of the invader [5-6].

Freshwater *Gammarus* amphipods are archetypal leaf shredders, having the functional role in river and lake ecosystems of processing major allochthonus energy inputs of fallen leaf litter from the riparian zone [7]. *Gammarus* spp. can form 85% (numerically and biomass) of taxa in riverine macroinvertebrate assemblages [8-9] and are 'keystone species' capable of influencing the population dynamics of other trophic levels [7]. They can be 'key' shredders, for instance individual *Gammarus* species can account for up to 16% of litter breakdown [7] and *Gammarus* spp. collectively up to 75% [8]. Litter processing via direct consumption, particle fragmentation and faeces production by shredders facilitates energy transfer between trophic levels, making these allochthonous energy inputs accessible to many other macroinvertebrate taxa [9]. In contrast, *D. villosus* is described as a 'predatory omnivore' and the feeding behaviour of this opportunistic invader is very flexible and its trophic function, seems to vary between ecosystems [10]. In some invaded systems it may be a relatively efficient and significant shredder / processor of leaf material [11-13], while in others it is a very poor leaf shredder [14]. Despite this variability, the continuing range expansion and associated predatory impact of *D. villosus* on resident shredder prey is an exemplar of a biological invasion that could dramatically affect ecosystem function in some invaded systems.

While consumptive effects of *D. villosus* eliminating shredder prey species would obviously affect energy processing and ecosystem function, MacNeil *et al*. [13] showed that NCEs might occur alongside CEs, as while many shredders were simply predated, the leaf shredding efficiency of survivors of the ongoing predation also decreased. The contribution of NCEs to biological invasion and its wider ecosystem level impacts could thus be even greater than previously appreciated [1,3]. We test whether exposure to just presence alone (with no possibility of physical interaction) of a predatory invader, could generate NCEs on naive prey behaviour. Specifically we assess if a NCE of *D. villosus* presence only (using caged confinement of the predator to isolate this) is evident on the shredding behaviour of three

92 different *Gammarus* prey species and which, alongside more obvious CEs such as injury by a
93 predator and predation itself, could undermine the prey species' functional role [7,10].

94

95 **Materials and methods**

96 We used a laboratory mesocosm approach to expose naïve prey to an invasive predator, which
97 allowed us to investigate NCEs without the possibility of accidental release of the invader.
98 During October 2003, *G. duebeni*, *G. pulex* and *G. tigrinus* were collected from three separate
99 locations in the Netherlands where *D. villosus* had not yet invaded (see Appendix 1 for details),
100 allowing simulation of initial interspecific contact and invasion [14]. Species were maintained
101 separately in holding tanks in the laboratory before being introduced to mesocosms [14]. Five
102 pre-weighed adults of either *G. duebeni*, *G. pulex*, *G. tigrinus* or *D. villosus* (mean wet weights
103 of each species ± SE = 44.2 ± 3.9 mg, 40.3 ± 3.7 mg, 24.2 ± 2.8 mg and 68.0 ± 4.1 mg
104 respectively) were introduced into mesocosms and these were able to swim freely and feed on
105 30 pre-weighed discs of 6mm diameter stream conditioned *Acer pseudoplatanus* leaf [14]. In
106 additional amphipod-free mesocosms submerged control leaf discs did not lose mass over the
107 experimental period (see Appendix 1). In half of the mesocosms a small plastic mesh cage
108 containing a single male *D. villosus* (standardised wet weight range 70-78 mg) was present
109 (caged *D. villosus* were not fed during the 4 days of the actual mesocosm experiment). This
110 placed the shredders in the chemical and visual presence of *D. villosus*, while preventing the
111 *D. villosus* from directly interacting with them. The remaining mesocosms were identical
112 except that *D. villosus* and cages were absent. We acknowledge inclusion of empty cages in *D.*
113 *villosus* 'free' mesocosms would have removed any potential 'cage effect' on amphipod
114 shredding activity and if resources had allowed we would have done this, but we do assume

any impact of a small empty plastic cage on the leaf shredding activity of five amphipods over a 4 day period, in a much larger mesocosm to be marginal at best.

Mesocosms were examined daily for 4 days and numbers of disks (to the nearest quarter of a disk) consumed each day recorded. When the number of remaining disks fell to 10 in any replicate, 10 further pre-weighed disks were added, ensuring material was always available in excess. Leaf shredding efficiency as leaf consumption per unit mass of shredder (mg wet weight leaf consumed per mg wet weight animal) in each separate 24hr period was estimated for each mesocosm [14]. There were n = 6 mesocosms for each shredder species and *D. villosus* treatment, such that n = 48 replicate mesocosms, with 192 observations of shredding efficiency across the 4 days.

Statistical methods

To determine the effects of predator treatment (*D. villosus*, absent or caged), shredder species (*G. duebeni*, *G. pulex*, *G. tigrinus* or *D. villosus*), day (1-4) and their interactions on shredding efficiency we used a linear mixed effects model. Due to the repeated measures nature of the data, we allowed random intercepts for replicates (mesocosms) to account for variation between them in shredding efficiency on day 1 and we also allowed random slopes to account for differences between them in changes in shredding efficiency across days 1-4. The analysis was implemented using the lme4 [15] and lmerTest [16] packages running under R version 3.4.1 [17], which in combination allow traditional *F* and *P* values to be calculated for fixed effects (see Appendix 2). Prior to analysis, data were $\log_{10}(x+1)$ transformed (as shredding efficiency could be <1).

Results

Not a single individual died during the 4 days. Shredding efficiency was, however, influenced by a 3-way interaction between presence of caged *D. villosus*, free swimming shredder species and day ($F_{6,176.09} = 11.74$, $P < 0.0001$) (Table 1; Figure 1). *G. duebeni* and *G. pulex* shredded more efficiently than *G. tigrinus*. Except for day 2 in the case of *G. pulex* and day 3 in the case of *G duebeni*, all *Gammarus* spp. showed reduced shredding efficiency in the presence of caged *D. villosus*. In contrast, free swimming *D. villosus* showed minimal amounts of shredding relative to the *Gammarus* spp. and this was unaffected by the presence of a caged conspecific. After 4 days, each *Gammarus* species showed lower shredding efficiency in the presence of caged *D. villosus* compared to the treatments where *D. villosus* was absent; *G. tigrinus*, the least efficient and smallest shredder, showing the greatest sensitivity to the presence of *D. villosus* (Figure 1).

Discussion

This study demonstrates an unappreciated and indirect impact of a biological invasion by a voracious predator; that the mere presence of an invader can influence resident prey behaviour, in this case the feeding efficiency of naïve residents. MacNeil *et al.*, [14] demonstrated strong CEs of uncaged *D. villosus* and indicated potential NCEs in surviving prey, with reduced shredding potentially reflecting injuries and loss of feeding time due to avoidance responses to predation-event cues (e.g. conspecific alarm cues or damaged conspecifics) and predator cues [9]. In contrast, the current analysis reveals that NCEs are not dependent on the occurrence of injuries or predation event cues. Rather, they resulted from the presence of caged *D. villosus* (unable to physically interact with prey), clearly demonstrating that prey responses to predator

cues caused by predator presence alone can lead to reduced shredding activity of all prey species.

The *Gammarus* spp. in our experiment had no prior exposure to *D. villosus* and would not have been under selection to respond to specific alarm cues from this predator [1]. Thus, this reduced shredding efficiency may reflect a generalized response to the presence of novel heterospecifics [18-19]. Here, the caged predator could have been detected via visual or semio-chemical cues [14, 19] but further studies would be needed to elucidate the exact mechanism driving this NCE. In addition, investigation of consumption of conspecifics in cages could produce larger behavioural responses from the prey [9]. Our data indicate that the elimination of resident prey may be driven by processes additional to direct predation, as at least to some degree, any reduced foraging efficiency may contribute to reduced survival and fecundity [1,7]. Our results also indicate that NCEs on functionally important prey species (key shredders [8-9] and keystone species [7]) may have repercussions at the ecosystem level. For example, recovery of stream communities following perturbation is dependent upon shredder facilitation, whereby recovery of other taxa and ecosystem processes is dependent on the recovery of shredders due to their contribution to energy flow [20].

The impact of NCEs on shredding activity seems unlikely to be uniform across locations or different naive prey species. The reduction in shredding efficiency was greatest in the least efficient shredder, *G. tigrinus*. This is unsurprising, as although *Gammarus* spp. are regarded as archetypal leaf shredders, all three species used here, are flexible omnivores and *G. tigrinus* is arguably the most predatory of the three [7]. Indeed, the ability of *Gammarus* to switch feeding strategy may generate a greater reduction in shredding efficiency, than would be the case for obligate herbivore-shredders, with a consequent greater reduction in energy processing.

Studies of invasion impacts have traditionally focussed on predation and biodiversity loss [2]. Here we demonstrate the potential for ecosystem level changes, simply due to the behavioural NCEs of mere exposure to a novel predator. A better understanding of the role of NCEs during biological invasions could enhance our ability to predict their progress and, in some cases, their wider ecosystem level ramifications.

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Table 1: Significance tests for fixed effects in the linear mixed model.

Effect	df	*F*	*P*
Predator treatment	1, 176.09	0.01	0.94
Shredder species	3, 176.09	570.51	<0.0001
Day	3, 176.09	0.31	0.58
Predator treatment x Shredder species	2, 176.09	5.12	0.002
Predator treatment x Day	3, 176.09	8.98	<0.005
Shredder species x Day	6, 176.09	10.56	<0.0001
Predator treatment x Shredder species x Day	6, 176.09	11.74	<0.0001

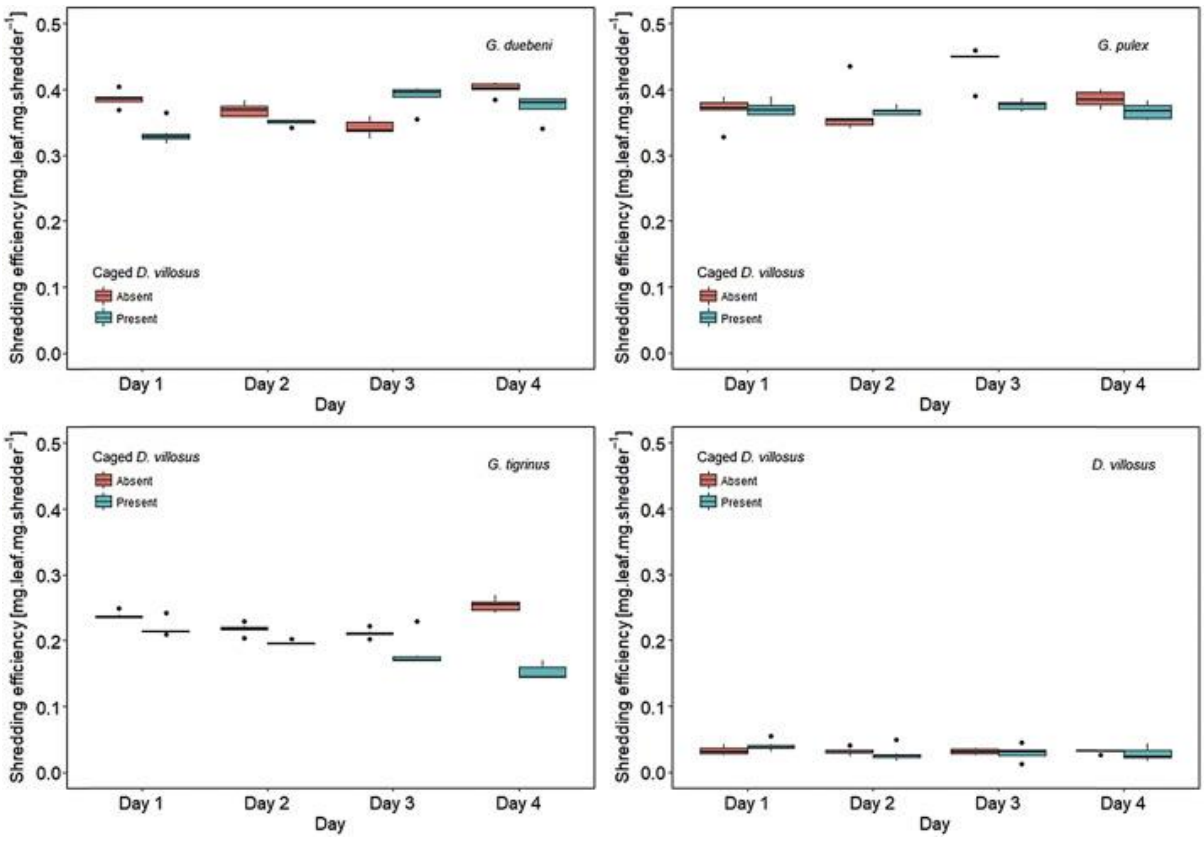

Figure 1: The three way interaction between caged *D. villosus* presence, free swimming shredder species and day, on shredding efficiency. Data for each species is shown on different panels. Lower and upper hinges show first and third quartiles respectively and whiskers represent the first and third quartiles – and + 1.5 interquartile ranges. Data points outside of these bounds are shown by dots. Untransformed raw data shown for clarity.

Appendix 1

METHODOLOGICAL DETAILS

Animal collection, holding tanks and mesocosms

D. villosus was collected from the Gouwzee lake (52°30'N 05°05'E), part of the Markermeer/IJsselmeer complex near Hoorn in the Netherlands (see MacNeil et al., 2008 for site details). *G. duebeni* was collected from a stream at Amstelmeer (52°53'N 4°53'E), *G. pulex* from a stream near Staverden (the Hierdense Beek; 52°17'N 05°44'E) and G. *tigrinus* from a lake at Oosterpoel (52°30'N 05°05'E) near Monnickendam. Each species was maintained separately in the laboratory in aerated tanks (60 x 40 x 10 cm deep) and acclimated in an equal mix of the 4 species' source waters (mean conductivity 850 μScm^{-1} for mixed water, range 707 – 890 μScm^{-1} for individual source waters) with substrate such as cobbles and pebbles, macrophytes, non-amphipod fauna such as mayflies, chironomids and snails and leaves from collection sites. All of the test animals can be maintained for several months under these conditions (C. MacNeil, personal observation). Animals were acclimatized for seven days in a light:dark cycle of 10:14 hours and a water temperature 17˚C, both these being appropriate for the time of year for the majority of streams sampled

Mesocosms consisted of plastic aquaria of 20 x 20 x 8 cm (length x width x height) were supplied with aerated, filtered mixed source water (17°C; mean ± SE dissolved oxygen 9.3 ± 0.24 mgl-1). Five clear glass pebbles (each 2 cm diameter) were placed in the aquaria, permitting animals to retreat into crevices, while still allowing observation.

Cages used to isolate *D. villous* in mesocosms were plastic mesh containers (8 x 7.5 x 4.5 cm) and were preconstructed aquarium shop products, used to isolate individual adult fish in tanks, either for treatment or to prevent adults eating eggs / fry. The mesh size was 1.8 mm. The cages were housed for 12 hours in a tank of the source water 'mix' used to fill mesocosms, before being used in mesocosm experiments.

Acer pseudoplatanus is an indigenous tree common at the collection sites and previous studies have shown its leaves to be highly palatable to amphipods. The 6-mm diameter disks were cut using a cork borer, avoiding the midribs (mean ± SE disk wet weight of 3.9 ± 0.5 mg, n = 200). Leaf disks in additional control aquaria (no animals present) showed negligible weight loss of <3% (range 0.9-2.8% of weight of initial disks added) over an 8 day observation period.

Appendix 2

STATISTICAL DETAILS

Model assumptions (normality of residuals and homogeneity of variance) were assessed via inspection of quantile and residual plots respectively. To improve the normality of residuals, data were log.10 (x+1) transformed. To allow for significance testing via F-tests, the degrees of freedom were estimated using the Satterthwaite approximation method. The analysis was coded as follows:

```
                                                    #requires packages:
                                                    #lme4
                                                    #lmerTest

data  <- read.csv
ID    <- data$ID
shred <- data$Shredder
dv    <- data$Predator
day   <- data$Day
eff  <- data$efficiency

log.eff <-log10(eff+1)                          #log x+1 transform
m1<-lmer(log.eff ~ shred*dv*day + (day|ID))   #LMM
```

```
anova(m1)                                   #Significance testing
```